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Nitric Oxide Synthase Expression and Nitric Oxide Production in Human Colon Carcinoma Tissue

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Background and Objectives: Nitric oxide (NO), the production of which is dependent on NO synthase (NOS), has been shown to contribute to various pathogeneses in cancer. The aim of this study was to determine whether inducible NO synthase (iNOS) is overexpressed in human colon carcinoma tissue, and whether NO is produced in tumor tissue.

Methods: We investigated iNOS mRNA expression in 24 human colon carcinoma tissue specimens by reverse transcription–polymerase chain reaction (RT-PCR). We then examined the expression of iNOS protein and nitrotyrosine, which indicates NO production in tissue, by immunohistochemistry. The possible immunosuppressive role of NO produced by colon carcinoma cells was analyzed in vitro.

Results: Semiquantitative RT-PCR analysis showed that iNOS mRNA expression in carcinoma tissues is elevated significantly compared to that in noncarcinoma tissue. Immunohistochemistry revealed that iNOS and nitrotyrosine are expressed strongly in carcinoma tissues. In vitro experiments showed that the supernatant from a culture of cytokine-treated colon carcinoma cells, which contained high levels of NO, significantly reduced the phytohemagglutinin (PHA)-stimulated, human lymphocyte proliferative response (60% of the control value).

Conclusions: In human colon carcinoma tissue, iNOS mRNA, protein, and NO products are overexpressed and may contribute to tumor-related immunosuppression. *J. Surg. Oncol. 1999;70:222–229.*

KEY WORDS: colon carcinoma; iNOS; NO; immunosuppression

INTRODUCTION

Nitric oxide (NO), which is thought to play a role in various physiological and pathological conditions [1,2], is produced by various cell types, including macrophages, endothelial cells, and certain carcinoma cells [2]. When large quantities of NO are generated locally, various pathological disorders, such as extensive tissue damage, can be caused [3]. NO reacts with superoxide ($O_2 \cdot^-$) at near diffusion-limited rates to form the strong oxidant $ONOO^-$. Nitration at the ortho position of tyrosine is a major product of $ONOO^-$ attack on proteins [4].

NO is produced by several NO synthase (NOS) types. Inducible NOS (iNOS) is calcium-independent and inducible by bacterial endotoxin (lipopolysaccharide, LPS) and inflammatory cytokines, such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ [1,5]. Increased expression of iNOS has been reported in several types of tumors. For example, squamous cell carcinoma of the head and neck, brain tumors, and breast tumors express high levels of iNOS [6–8]. It has also been reported that gynecological tumors express higher

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TABLE I. Clinical Profiles of 24 Colon Cancer Patients

Case	Age	Sex	Cancer site	Dukes′ stage	Histology[a]
1	69	M	Sigmoid colon	A	Well differentiated
2	67	M	Descending colon	D	Well differentiated
3	57	F	Ascending colon	D	Moderately differentiated
4	63	F	Ascending colon	C	Well differentiated
5	63	F	Sigmoid colon	C	Mucinous
6	44	M	Ascending colon	C	Moderately differentiated
7	72	F	Ascending colon	D	Poorly differentiated
8	67	F	Sigmoid colon	B	Well differentiated
9	59	F	Rectum	C	Moderately differentiated
10	73	F	Rectum	B	Moderately differentiated
11	67	M	Rectum	C	Moderately differentiated
12	56	M	Rectum	A	Well differentiated
13	43	F	Rectum	B	Well differentiated
14	72	M	Ascending colon	A	Well differentiated
15	42	M	Sigmoid colon	A	Well differentiated
16	77	M	Rectum	A	Moderately differentiated
17	63	F	Rectum	D	Moderately differentiated
18	61	F	Sigmoid colon	A	Moderately differentiated
19	72	F	Transverse colon	A	Undifferentiated
20	55	M	Sigmoid colon	A	Moderately differentiated
21	63	M	Sigmoid colon	A	Well differentiated
22	85	F	Descending colon	C	Well differentiated
23	51	F	Sigmoid colon	A	Well differentiated
24	53	F	Descending colon	C	Well differentiated

[a]Poorly differentiated: poorly differentiated adenocarcinoma; moderately differentiated: moderately differentiated adenocarcinoma; well differentiated: well-differentiated adenocarcinoma; Undifferentiated: undifferentiated adenocarcinoma; mucinous: mucinous adenocarcinoma.

levels of iNOS than do nontumor tissues [9]. Despite the findings reported above, the expression of iNOS in colon carcinoma tissue remains controversial. Recently, Takahashi et al. [10] reported increased expression of iNOS and endothelial NOS in rat colon tumors induced by azoxymethane. However, Moochhala et al. [11] reported that human colon carcinoma tissue expresses less iNOS than does the normal colon epithelium.

Upregulation of iNOS activity is associated with production of large amounts of NO. Because NO has been shown to inhibit T-cell proliferative response [12], NO produced in tumor tissue may interfere with the action of lymphocytes against tumor cells and cause immunosuppression. Recently, Young et al. [13] demonstrated that NO is associated with tumor-induced immunosuppression using in vivo animal model. However, the role of NO in immunosuppression of human cancer is less clear.

In the present study, we investigated whether colon carcinoma tissue expressed iNOS and produced NO. We also addressed the possible role of NO in local immunosuppression of human colon carcinoma tissue.

MATERIALS AND METHODS

Tissue Collection

Twenty-four colon cancer patients were entered into the study (Table I). We obtained informed consent from all patients before including them in the study. Both carcinoma and noncarcinoma colon mucosa tissue specimens were obtained from the patients. Tissue samples from each site either were fixed by immersion in neutral-buffered formalin for both histological diagnosis and immunolabeling studies or were freeze-clamped and stored at −80°C for reverse transcription–polymerase chain reaction (RT-PCR) analysis for iNOS.

Tumor Cells

The DLD-1 cell line, which is originated from human colon carcinoma cells [14], was obtained from the American Type Culture Collection (Rockville, MD). DLD-1 cells were maintained in Dulbecco's Modified Eagle Medium (D-MEM; Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum (FBS; Filtron, Victoria, Australia) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C.

Lymphocyte Collection

Blood was obtained from normal human volunteers by venipuncture into syringes containing heparin. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by density gradient centrifugation at 1,550 rpm for 30 min on Histopaque-1077 (Sigma Chemical, St. Louis, MO). PBMC at the plasma/Histopaque interface were removed, washed three times in D-MEM, and then resuspended in D-MEM supplemented with 10%

FBS in a 25-cm^2 tissue culture flask. The cells were allowed to attach to the tissue culture flask for a minimum of 3 hr at 37°C in a humidified atmosphere containing 5% CO_2 in air. Nonadherent cells, such as lymphocytes, were harvested and used for subsequent assays. Cells were counted with a hemocytometer using trypan blue exclusion, and viability was consistently greater than 98%.

Reagents

Recombinant human IFN-γ was supplied by Shionogi Pharmaceutical (Osaka, Japan). Recombinant human IL-1β was supplied by Otsuka Pharmaceutical (Tokushima, Japan). LPS and N^G-monomethyl-L-arginine (NMMA) were purchased from Sigma Chemical.

RT-PCR and Semiquantitative Measurement

Total RNA was extracted from the tissue samples by the guanidinium thiocyanate phenol extraction method as described previously [15]. Total RNA (1 μg) was converted to cDNA with Superscript II and random hexamers (Life Technologies, Rockville, MD). To assess the amount of iNOS-specific messenger RNA (mRNA) in each sample, we performed PCR for both iNOS and a constitutively expressed housekeeping gene, hypoxanthine phosphoribosyltransferase (HPRT). The oligonucleotide primers were: iNOS sense GCCTCGCTCTGGAAAGA and iNOS antisense TCCATGCAGACAACCTT, which amplify a 499-bp product [14], and HPRT sense CGAGATGTGATGAAGGAGATGG and HPRT antisense GGATTATACTGCCTGACCAAGG, which amplify a 313-bp product [16], (Nisshinbo, Tokyo, Japan). Amplification was initiated by 5 min of denaturation at 95°C for 1 cycle followed by 35 cycles of 95°C for 2 min, 58°C for 1 min, and 72°C for 2 min. To validate our PCR cycles for each transcript, we quantified the PCR products with serial dilutions of cDNA. Starting with cDNA in the 2-μl diluted solution, which was originally from 50 ng of total RNA, 10-fold serial dilutions were then generated for the sample expressing the most intense band. A linear correlation was observed between the dilution logarithms and expression amounts (data not shown).

The gelphotographs were scanned on a Scan Jet 4C/T (Hewlett Packard, San Diego, CA). Densitometry was performed using the Adobe Photoshop software package (Adobe Systems, Mountain View, CA) and NIH image 1.60 software (NIH Division of Computer Research and Technology) and plotted with Statview J-4.5 software (Abacus Concepts, Berkeley, CA) on a Macintosh Centris computer (Apple Computer, Cupertino, CA). To ascertain the minimum variance from the photograph in this procedure, each PCR product from the human colon carcinoma cell line DLD-1 cells was included on the gel as an internal control. The amount of mRNA expression was calculated as the ratio of the samples to DLD-1 cells on the same photograph. Each quantity of mRNA expression was then corrected by that of the corresponding HPRT mRNA expression.

Immunohistochemistry

Tissue samples were fixed in 10% neutral-buffered formalin (Wako Pure Chemical Industries, Osaka, Japan), embedded in paraffin, sectioned (4- to 5-μm slices), and deparaffinized. Slides were immersed first in 0.3% hydrogen peroxide for 30 min and then in normal goat serum (1.5%) for 20 min to block endogenous peroxidase activity and nonspecific binding sites, respectively. Immunostaining was performed with a rabbit polyclonal IgG specific for iNOS (Wako Pure Chemical Industries) at a dilution of 1:500 at 37°C for 1 hr. A second immunostaining was performed with a rabbit polyclonal antinitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY). Nitrotyrosine, a stable end product of the nitration of tyrosine residues, can be used as a marker for $ONOO^-$ and other nitrating species [17]. The sections were treated with biotinylated secondary antibodies at a dilution of 1:200 (Nichirei, Tokyo, Japan), and the antibody-binding sites were visualized by avidin-biotin peroxidase complex solution and 3,3′-diaminobenzidine (Wako Pure Chemical Industries).

In Vitro Stimulation of DLD-1 Cells

To examine colon carcinoma cell expression of iNOS, we stimulated DLD-1 cells with LPS, IL-1β and IFN-γ. To measure NO production, tumor cells (5×10^5 cells/well in 2.0 ml of D-MEM 10% FBS) were cultured in a 24-well culture dish (Nunc, Roskilde, Denmark) with or without LPS (100 ng/ml), IL-1β (1 ng/ml), and IFN-γ (250 U/ml) for 24 hr at 37°C in a CO_2 incubator (5% CO_2 + 95% air). The accumulation of nitrite (NO_2^-) and nitrate (NO_3^-) in the culture media was then measured. In some experiments, the NOS inhibitor NMMA was added to the LPS and cytokine-treated DLD-1 cell cultures.

Measurement of NO Production by Tumor Cells (Griess Method)

NO generation was determined by measuring the sum of NO_2^- and NO_3^- concentrations in the culture supernatant as described previously [14]. Briefly, 80 μl of culture supernatant was mixed with 80 μl of the Griess reagent (Dojindo, Kumamoto, Japan) and then incubated for 10 min at room temperature in a 96-well microplate. The optical density was measured at 570 nm using an automatic plate reader (Easy Reader, SLT-labinstruments, Grodig, Austria). NO_2^- and NO_3^- concentrations were determined from a standard curve prepared with known concentrations of $NaNO_2$ and $NaNO_3$. D-MEM contained very little NO_3^- (<1 μM); therefore, the background of each sample could be ignored.

Lymphocyte Proliferation Assay

Lymphocytes (5×10^5 cells/well) were cocultured for 72 hr with DLD-1 cells (5×10^5 cells/well) in 24-well culture dishes (Nunc). Population were separated by Intercell (pore size 0.45 μm, Kurabo, Osaka, Japan). The lymphocytes were removed, and the phytohemagglutinin (PHA)-induced proliferation was measured using a [^{3}H]-thymidine (New England Nuclear, Boston, MA) incorporation assay. The assay was performed in a 96-well round bottomed plate (Nunc). Lymphocytes were seeded in triplicate in 200-μl final volume (1×10^5 cells) in complete medium with PHA (0.5 μg/ml). Plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air for 60 hr before addition of a radioactive thymidine label. After [^{3}H]-thymidine incorporation for 12 hr, the cells were trapped onto glass fiber filter paper in a cell harvester (LKB-Wallac, Turku, Finland) by vacuum filtration. The individual filters were then counted with a scintillation counter (LKB-Wallac).

Statistical Analysis

Statistical significance was calculated with Student *t*-test, and $P < 0.05$ was considered statistically significant. All results are shown as mean ± standard error.

RESULTS

iNOS mRNA Expression of Clinical Samples

We used DLD-1 cells, which are known to express iNOS, to determine the appropriate condition for iNOS RT-PCR. The amplification curve for iNOS mRNA is shown in Figure 1. PCR product was amplified in proportion to the PCR cycles at 10 to 50 cycles, but at more than 45 cycles, the curve changes significantly to indicate a much slower rate of increase. Therefore, we selected 35 cycles for PCR detection of iNOS mRNA expression. We then examined iNOS mRNA expression in colon carcinoma tissues and compared the findings with those from adjacent noncarcinoma tissues from our patients. The data are summarized in Figures 2 and 3. Although the frequency of iNOS expression between carcinoma and noncarcinoma tissue specimens did not differ significantly (positive %; carcinoma 95.8%, noncarcinoma 87.5%), the relative expression of iNOS mRNA as determined by semiquantitative RT-PCR was significantly greater in carcinoma tissue than in noncarcinoma tissue (0.729 ± 0.058 vs. 0.407 ± 0.063, $P < 0.001$) (Fig. 3).

iNOS Protein and Nitrotyrosine Expression of Clinical Samples

We examined iNOS protein expression in human colon carcinoma tissues. The clinical profiles of patients with colon cancer included in this study are shown in Table I. We first assessed iNOS protein expression in colon carcinoma tissue in comparison to noncarcinoma tissue from the same patients. The anti-iNOS immunoreactivity in colon carcinoma tissue was positive in all tumor tissue specimens from the 24 patients tested. A representative section is shown in Figure 4. Immunoreactivity throughout the tissue sections ranged from well- to poorly differentiated and undifferentiated adenocarcinoma cells. Some regions of the carcinoma showed strong immunoreactivity, while others showed poor reactivity. In contrast, in normal colon mucosal tissue, iNOS protein expression was barely detectable in epithelial and stromal cells. In some stromal tissue specimens from normal colon tissue, iNOS was weakly positive in the macrophages.

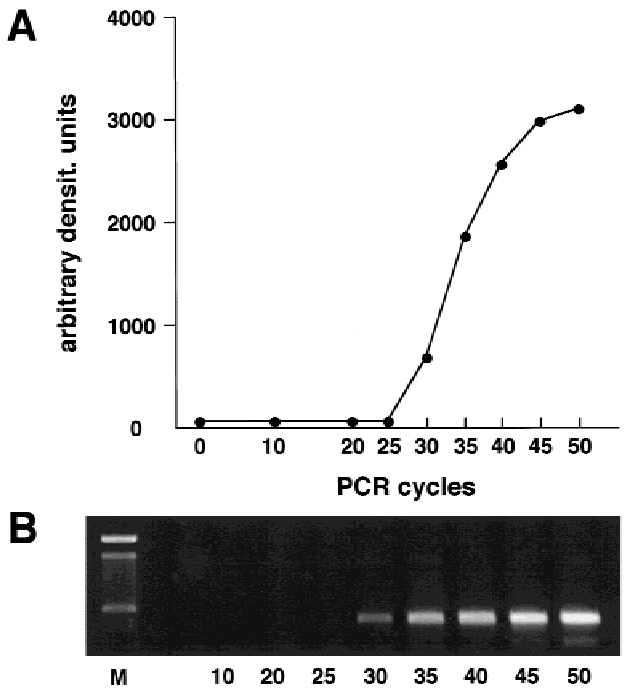

Fig. 1. **(A)** PCR product amplification curve for iNOS mRNA from DLD-1. **(B)** Ethidium bromide staining of PCR products from different PCR cycles.

Immunoperoxidase staining of tumor serial sections demonstrated specific binding of the nitrotyrosine antibody. The cytoplasms and nuclei of tumor cells were positive for nitrotyrosine staining (Fig. 5A). Nitrotyrosine staining was also pronounced in stromal cells around the tumor cells, especially in the area between the interstitial space and the tumor cell nest (Fig. 5B). In noncarcinoma tissue, weak staining of nitrotyrosine was observed only in stromal cells (data not shown).

Immunosuppressive Effect of NO Produced by Carcinoma Cells

An in vitro study revealed that LPS and cytokine (IL-1β and IFN-γ) treatment increased iNOS mRNA expression in DLD-1 cells (Fig. 6). We then measured NO production by cytokine-stimulated DLD-1 cells. The cul-

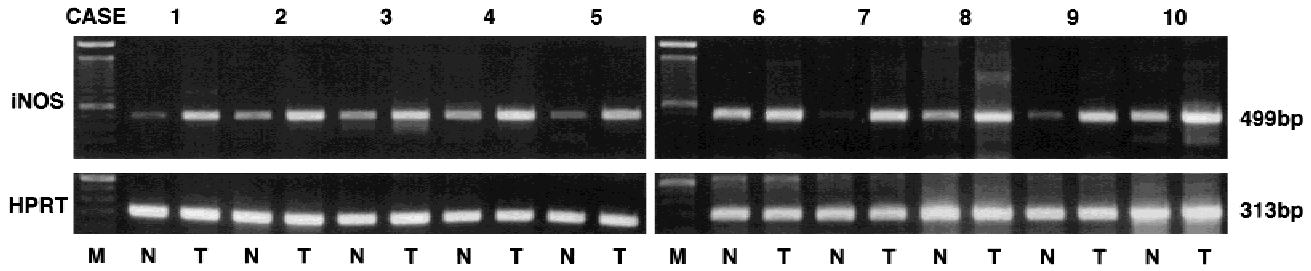

Fig. 2. Representative iNOS and HPRT mRNA expression in colon carcinoma tissues analyzed by RT-PCR. PCR products were separated on an agarose gel and stained with ethidium bromide. Lane M: molecular marker; lane N: noncarcinoma tissue; lane T: carcinoma tissue.

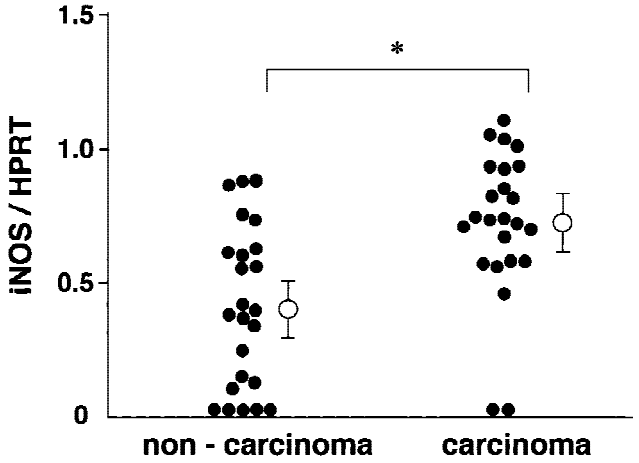

Fig. 3. Semiquantitative RT-PCR of iNOS mRNA expression in total RNA extracted from colon carcinoma specimens and from their paired control noncarcinoma samples. Data are expressed as the ratio of iNOS mRNA/HPRT mRNA. Values (mean ± standard error) represent the ratio of iNOS/HPRT calculated from the arbitrary densitometric units.

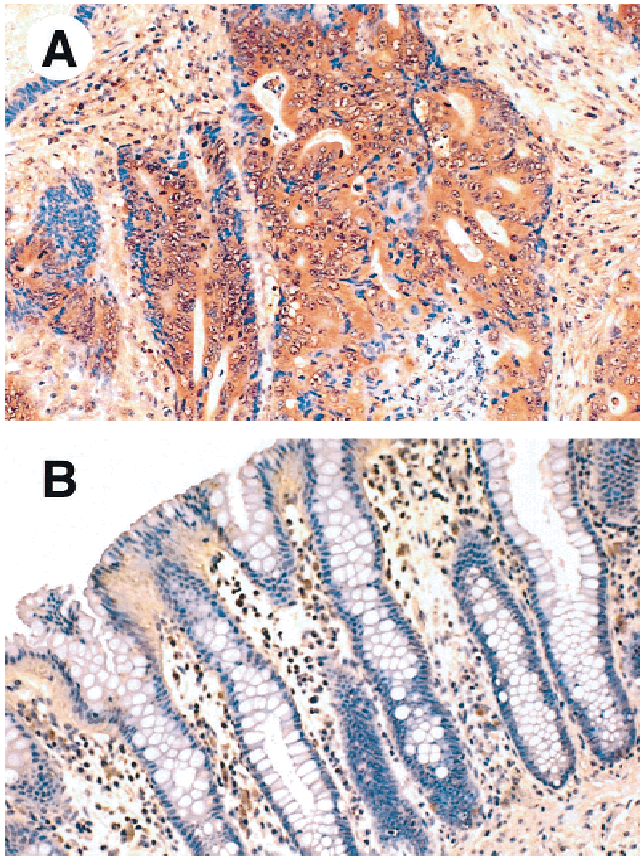

Fig. 4. Immunohistochemical staining of iNOS in colon carcinoma tissue. **(A)** Carcinoma tissue. Immunoreactive iNOS is expressed strongly in carcinoma tissue. **(B)** Noncarcinoma tissue. Weak iNOS immunoreactivity for iNOS was observed in the normal tissue stroma but not in the normal epithelium.

ture supernatant from LPS and cytokine-treated DLD-1 cells was determined to contain high levels of NO (55.0 ± 2.3 μM). The increases in NO levels were inhibited by addition of NMMA (22.1 ± 1.5 μM), suggesting that the LPS and cytokine-induced NO production by DLD-1 cells was a specific event ($P < 0.01$) (Fig. 7).

Lastly, we examined whether the NO produced by DLD-1 cells affects normal lymphocyte function. The lymphocytes and DLD-1 cells were cocultured. The lymphocytes were then removed, and the PHA-stimulated proliferation response was tested. The PHA-stimulation index of lymphocytes cocultured with cytokine-added DLD-1 cells was significantly less than that of lymphocytes cocultured with nontreated DLD-1 cells (3.70 ± 0.20 vs. 6.13 ± 0.23, $P < 0.01$; Fig. 8). NMMA inhibited the suppressive effect of LPS- and cytokine-treated DLD-1 cells on lymphocyte proliferation (PHA-index 5.17 ± 0.17), indicating that NO is one immunosuppressive factor produced by colon carcinoma cells.

DISCUSSION

Recently NO has been shown to play a role in tumor actions, such as angiogenesis, regulation of microcirculation, and cellular injury [18–20]. In this study, we showed that human colon carcinoma tissue expresses more iNOS mRNA and protein than do noncarcinoma tissue, suggesting that NO levels may be greater in carcinoma tissues than in noncarcinoma tissues. In vitro production of NO and related oxidants have been difficult to demonstrate so far. One stable product of $ONOO^-$ attack on proteins is nitrotyrosine, which comes from the addition of a nitro group (NO_2) to the ortho position of tyrosine. In the present study, nitrotyrosine staining was

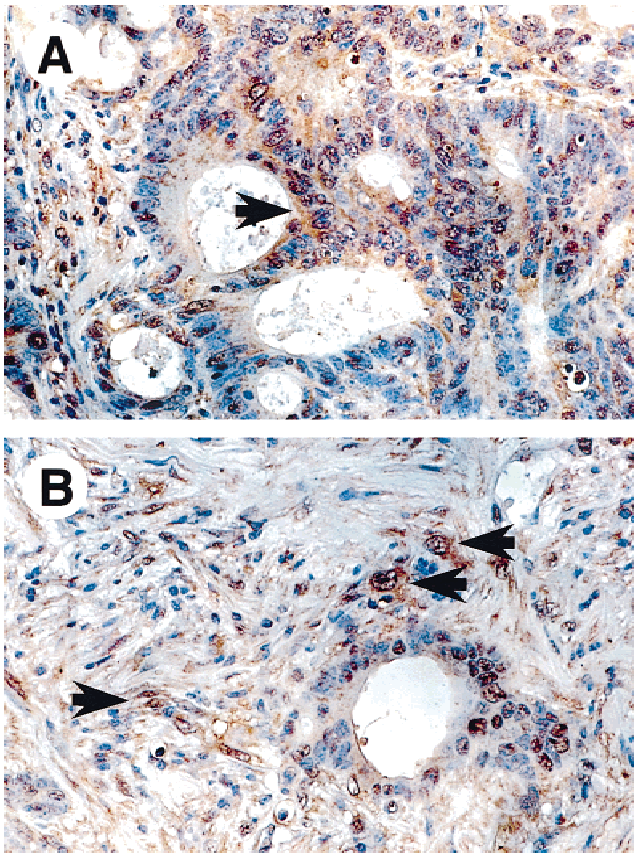

Fig. 5. Immunohistochemical staining of nitrotyrosine in colon carcinoma tissue. Brown reaction product indicates specific antibody binding. **(A)** Carcinoma cells. Immunostaining was strongly positive in cytoplasms and nuclei of carcinoma cells (arrow). **(B)** Stromal tissue. Stromal cells (macrophages, fibroblasts) surrounding carcinoma cells are also positive for nitrotyrosine (arrows).

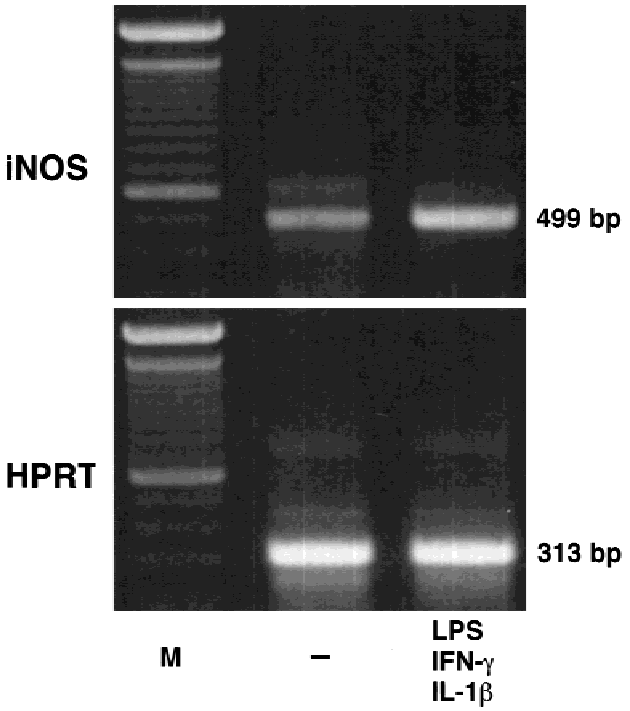

Fig. 6. iNOS and HPRT mRNA expression by DLD-1 cells. DLD-1 cells (1×10^6) were treated or not treated with LPS, IL-1β and IFN-γ. Lane M: molecular marker.

prominent in tumor tissue that was positive for iNOS staining. These results are consistent with recently published reports [21,22].

Despite the findings discussed above, the expression of iNOS in colon carcinoma tissue remains controversial. Moochhala et al. [11] reported that human colon carcinoma tissue expresses less iNOS than does the normal colon epithelium. On the other hand, Ambs et al. [23] have recently reported overexpression of iNOS and nitrotyrosine formation in human colon adenomas and carcinomas. These findings were consistent with our results, which showed overexpression of iNOS in colon carcinoma cells. However, several points differ between Ambs et al.'s results and ours. First, their findings about iNOS were mainly dependent on biochemical activity and immunohistochemistry of iNOS but not mRNA. Second, although they reported that nitrotyrosine formation was positive in macrophages, we showed that nitrotyrosine was positive in not only tumor stromal cells, such as macrophages, but also in tumor cells. These differences among the studies may be partly due to tissue fixation conditions, assay conditions, or the polyclonal antibody used.

NO may affect several pathological conditions. Recent studies have suggested that NO plays a role in increased tumor blood flow, edema, and vascular permeability [24]. These tumor features are seen in pathologically high-grade tumors of the colon and rectum. We also found some correlations between the amount of iNOS expression and pathological parameters, such as blood vessel counts and lymphatic invasion in the colon carcinoma tissues (data not shown). This suggests that NO may affect tumor progression. The mechanism by which iNOS expression in tumor tissue is regulated is less understood. Advanced-stage colon carcinoma tissues can contain LPS produced by Gram-negative bacteria and cytokines from tumor infiltrating mononuclear leukocytes. Therefore, it is possible that LPS and cytokines may thus induce iNOS expression by colon carcinoma cells in vivo.

Using in vitro study, we confirmed that colon carcinoma cell lines can produce NO and suppress PHA-stimulated lymphocyte proliferation. And NMMA inhibited the suppressive effect. These results suggest that large amounts of NO produced in colon carcinoma tissue may influence antitumor lymphocyte reaction and cause immunosuppression. Therefore, NO inhibition treatment of colon carcinoma may increase the antitumor immune response. The mechanism by which NO inhibits the lym-

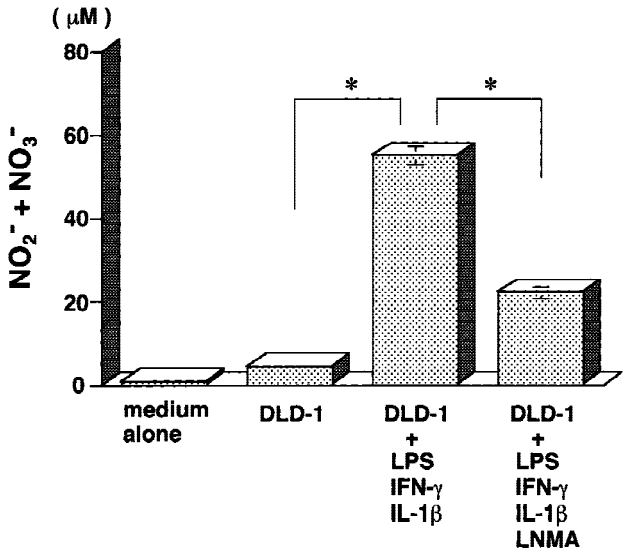

Fig. 7. NO production by LPS and cytokine-treated DLD-1 tumor cells (5×10^5 cells/well in 2.0 ml of D-MEM/10% FBS). Left column is medium alone. DLD-1 cells were cultured in medium alone; LPS and cytokines (IL-1β, IFN-γ); or LPS, cytokines, and NMMA. NO production in the culture supernatant was assessed after 24-hr incubation. Each result is the mean ± standard error of triplicate cultures form one of three representative experiments that had similar results. Asterisk denotes $P < 0.01$.

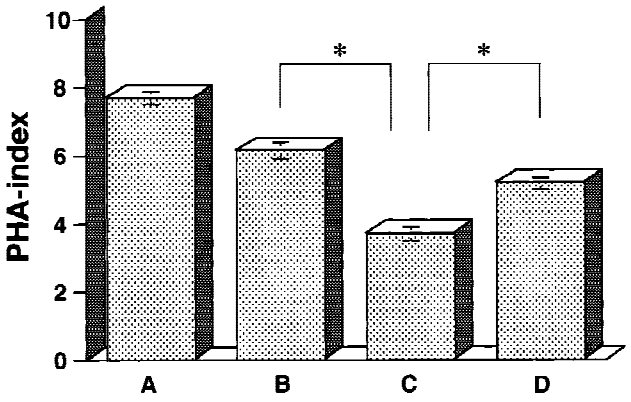

Fig. 8. Influence of supernatant from LPS and cytokine-treated tumor cells on PHA-stimulated lymphocyte proliferation. The lymphocytes were cocultured with DLD-1 cells for 72 hr as described in text. The lymphocytes (1×10^5 cells) were separated, stimulated with 0.5 μg/ml of PHA for 60 hr, and then pulsed with [^{3}H]-thymidine for 12 hr. The PHA index was calculated as follows: the PHA index = thymidine uptake by PHA-stimulated lymphocytes/thymidine uptake by nonstimulated lymphocytes (mean ± standard error). **(A)** Lymphocytes alone. **(B)** Lymphocytes cocultured with untreated DLD-1 cells. **(C)** Lymphocytes cocultured with LPS and cytokine-treated DLD-1 cells. **(D)** C plus NMMA. Asterisk denotes $P < 0.05$.

phocyte response remains unclear. Several studies have shown that NO causes DNA oxidation, deamination, and strand-break formation [25,26]. NO reacts with superoxide to form $ONOO^-$, which is a powerful one- and two-electron donor capable of oxidizing and nitrating target molecules and is lethal to cells in vitro and in vivo [27,28]. Thus, NO may cause mitochondrial dysfunction in lymphocytes, especially in the presence of superoxide.

In conclusion, iNOS mRNA, protein, and NO production are overexpressed in human colon carcinoma tissue. And the high NO production may be associated with such pathophysiological processes as immunosuppression in colon cancer. The regulation of NO production by agents, such as NOS inhibitors, may be useful therapeutically in the treatment of colon cancer.

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